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(54) Title: CYTOKINE EXPRESSED BY DIA/LIF-DEFICIENT EMBRYONIC STEM CELLS FOR THE INHIBITION OF DIFFERENTIATION

(57) Abstract

A novel cytokine having the capacity to inhibit differentiation of embryonic stem (ES) cells is provided. The cytokine is designated ESRF and is characterised by the capacity to inhibit differentiation of ES cells (i) in the absence of DIA/LIF and (ii) in the absence of cytokines which act through gp 130 and (iii) in the absence of interaction with gp 130 and (iv) in the absence of interaction with LIF receptor. The invention further provides methods of deriving and propagating ES cells, ES cells *per se* and cell lines useful in deriving and assaying novel factors.

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CYTOKINE EXPRESSED BY DIA/LIF-DEFICIENT EMBRYONIC STEM CELLS FOR THE INHIBITION OF DIFFERENTIATION

Field of the Invention

This invention relates to substances having the capacity to inhibit differentiation of embryonic stem (ES) cells. The invention further provides methods of deriving and propagating ES cells, ES cells *per se* and cell lines useful in deriving and assaying novel factors.

Background to the Invention

Embryonic stem cells are the archetypal stem cell, being capable both of unlimited self-renewal and of differentiating to form the whole gamut of cell types found in the adult animal. Such stem cells are described as pluripotential as they are capable of differentiating into many cell types. These cells will participate fully in normal embryogenesis following reintroduction into blastocysts and can contribute functional differentiated progeny to all somatic tissues and to the germ line. ES cells can also be induced to differentiate into a wide variety of cell types in culture, recapitulating *in vitro* processes responsible for tissue diversification in the developing embryo. ES cells therefore provide a unique system for the analysis of factors that control early embryonic growth and differentiation.

ES cells have the additional property of being able to participate fully in normal embryogenesis following reintroduction into host embryos and contribute functional differentiate progeny to all somatic tissues and to the germ line. Their ability to form gametes allows ES cells to be used as a means of transmitting genetic modifications into animals. This is exploited for gene discovery and mutation though gene trapping strategies and most importantly for precise gene alteration via gene targeting.

In general, when required for research purposes or for medical use, stem cells have to be isolated from tissue samples by various fractionation procedures, but even after careful segregation of cell types, these stem cell preparations consist of mixed cell types and while enriched for stem cells, include high proportions of differentiated cells which are not categorised as stem cells.

Furthermore, most stem cells cannot be grown readily in culture and when attempts are made to culture stem cells, and more particularly to maintaining established lines of ES cells, the cells being cultured (which ordinarily contain a mixed population of cell types) grow at different rates and stem cells rapidly become overgrown by non-stem cell types. An exception is that embryonic stem cells from two specific strains of mice (129 and Black 6) can be cultured *in vitro*. Thus established lines of embryonic stem cells can be obtained by culturing early (3½ day) embryonic cells from murine strain 129 and Black 6, or hybrids thereof.

Proven embryonic stem cells with the capacity for germ line transmission have to date only been established from specific inbred strains of mice (notably strains 129 and C57BL/6). They are derived by culturing early embryonic cells in the presence of a feeder layer of embryonic fibroblasts and/or of cytokine leukaemia inhibitory factor (LIF) or related cytokines which act through the signal transducer glycoprotein 130 (gp130) (Yoshida *et al.*, 1994). In the absence of a source of LIF the stem cells differentiate and cannot be propagated. By contrast, ES cells cultures in the presence of DIA/LIF or of a feeder layer of DIA/LIF-producing cells maintain their proliferative capacity, retain characteristic stem cell morphology, and express stem cell markers such as alkaline phosphatase, stage-specific embryonic antigen-1 and the stem cell-specific transcription factor Oct-3/4. ES cells expanded in the presence of LIF remain pluripotential and competent to produce germline chimaeras when reintroduced into mouse blastocysts (Nichols *et al.*, 1990; 1994).

It is known that cytokines play an important role in the maintenance of the pluripotential phenotype of ES cells *in vitro*. Thus the investigation of factors which regulate proliferation and differentiation of ES cells led to the purification of a glycoprotein of apparent molecular weight 45 kDa named "Differentiation inhibiting activity" or DIA. This is identical to the cytokine "Myeloid leukaemia inhibitory factor" or LIF. DIA/LIF acts to sustain the self-renewal of undifferentiated ES cells and thereby allows their propagation *in vitro*. ES cells maintained in the presence of this factor retain their full development potential. Moreover ES cells can be established *de novo* by direct culture of blastocysts in medium supplemented with DIA/LIF. (The term "cytokine" as used herein refers to any substance which acts from outside a cell and is capable of affecting cell survival and/or growth and/or differentiation. Primarily the term denotes a proteinaceous factor capable of inhibiting differentiation of ES cells).

In the absence of a source of DIA/LIF, the stem cells differentiate and cannot be propagated. By contrast, ES cells cultured in the presence of DIA/LIF or of a feeder layer of DIA/LIF-producing cells maintain their proliferative capacity, retain characteristic stem cell morphology and express stem cell marker proteins such as alkaline phosphatase and stage-specific embryonic antigen-1 (SSEA-1) (Williams et al., 1988; Smith et al., 1988). Most significantly, ES cells expanded in the presence of DIA/LIF remain pluripotential and competent to produce chimeras when reintroduced into mouse blastocysts (Nichols et al., 1990; Pease et al., 1990).

Further study has revealed that undifferentiated ES cells express low levels of mRNA which gives rise to the matrix-localised form of DIA/LIF, whereas differentiated cells express relatively high levels of both soluble and matrix-associated DIA/LIF. It has been proposed that the enhanced production of DIA/LIF by newly differentiated cells may provide a mechanism for regulating the balance between differentiation and self-renewal in stem cell populations. The physiological importance of DIA/LIF has been established by the finding that homozygous DIA/LIF-deficient female mice are sterile due to an incapacity to support embryo implantation. However, DIA/LIF -/- embryos are viable. This observation suggests

that other factors may compensate for the absence of DIA/LIF expression during early development. It has recently been shown that a number of cytokines, including DIA/LIF, interleukin-6 (IL-6), ciliary neurotrophic factor (CNTF) and oncostatin M (OSM), share the same effector molecule gp130. This presumably underlies the considerable overlap in biological activities reported for these factors. Activation of gp130 signalling processes by any of these cytokines is sufficient to support ES cell self-renewal. However, it is not yet clear which, if any, of these molecules plays a role in the maintenance of stem cell renewal early during embryogenesis or whether other factor(s) are involved in this process.

There has developed a pressing need to isolate and maintain *in vitro* embryonic stem cells from other murine strains and more especially from other species including other laboratory animals (eg. rats, rabbits and guinea pigs), domesticated animals (eg. sheep, goats, horses, cattle and pigs) and primates including human. The demand for this is twofold. Firstly to extend gene targeting and other sophisticated genome manipulation technologies such as chromosome modification (Smith A.J.H. *et al.*, 1995) into other species, notably the rat, which is the experimental model of choice for the pharmaceutical industry, and the pig, which requires genetic adaptation for use in xenotransplantation. The second need is for the development of human ES cells as a universal source of donor cells for transplantation. However, no known techniques exist for generating and maintaining established lines of true embryonic stem cells other than from the mouse strains noted above and from hybrids thereof. Also, the only known method of maintaining ES cells used either DIA/LIF or related cytokines as described above or feeder layers as a source of such cytokines. A further disadvantage of the known ES derivation methods is that expression of gp130 by donor embryo cells is essential.

Statement of Prior Art

A paper entitled "Pluripotent Embryonic Stem Cells from the Rat Are Capable of Producing Chimeras", Iannaccone, P.M. *et al.*, Dev. Biol. 163, 288-292 (1994) describes a cell line designated RESC-01 which is stated in the paper to consist of "diploid rat embryonic stem cells". Further the RESC-01 cell line is stated to be capable of being "used to produce chimeras by injection into rat blastocysts". The aforementioned paper does not characterise the RESC-01 cell line by reference to its karyotype and in the absence of such characterisation, the data presented is insufficient to support the allegation that the RESC-01 cell line consists of rat ES cells. It is understood from subsequent disclosed information (personal communication, not published) that the RESC-01 cell line has a mouse karyotype, not rat. It is apparent therefore that Iannaccone *et al*'s stated aim "The ability to establish a stem cell population from the rat and make chimeras with them is the first step toward providing an important addition to the repertoire of genetic manipulation techniques in mammals" has hitherto been unfulfilled.

Further a paper entitled "Isolation of a primate embryonic stem cell line ", Thomson J.A. *et al.*, Proc. Natl. Acad. Sci. U.S.A. describes a cell line designated R278.5 which is stated to be an ES cell line. However although R278.5 possess a number of the characteristics of ES cells, it has not been shown to be a true ES cell line by at least one of the following criteria:

(i) extensive contribution to chimeras without tumour formation,

(ii) reconstitution of host tissues,

(iii) contribution of functional gametes to chimeras and generation of offspring.

Summary of the Invention

In order to define the contribution of DIA/LIF *in vitro* and establish an assay system for detecting factors which can regulate ES self-renewal, we have generated ES cells in which both copies of DIA/LIF gene have been deleted. We report here that differentiated DIA/LIF-deficient ES cells synthesize a novel soluble factor which inhibits ES cell differentiation. This factor is distinct from previously characterised ES cell maintenance factors and most significantly acts independently of direct activation of gp130. ES cells have also been generated which lack LIF receptor. The LIF-negative and LIF receptor-negative cells (LIF(-) and rLIF(-)) are additionally useful in assay procedures which form further aspects of the invention.

According to one aspect of the present invention, there is provided a novel cytokine designated ESRF (ES cell Renewal Factor) and characterised by the capacity to inhibit differentiation of ES cells in the absence of DIA/LIF and without direct interaction with gp130.

More specifically, there is provided a cytokine designated ESRF and characterised by the capacity to inhibit differentiation of ES cells (i) in the absence of DIA/LIF and (ii) in the absence of cytokines which act through gp130 and (iii) in the absence of interaction with gp130. The capacity to inhibit differentiation of ES cells (iv) in the absence of interaction with LIF-receptor may further be used to characterise the cytokines of the invention.

The cytokine of the invention may be further characterised by features selected from the following:

- being distinguishable from DIA/LIF
- being distinguishable from IL-6/sIL-6R
- being distinguishable from CNTF
- being distinguishable from oncostatin M

- being distinguishable from cardiotrophin-1

taken individually or in combination. ("IL-6/sIL-6R" is an abbreviation of "interleukin-6/soluble interleukin-6 receptor").

The cytokine designated ESRF herein is intended to include ESRF derived directly or indirectly from any mammalian species, including laboratory animals (i.e. rodents), domestic and farmyard animals (e.g. dogs, cats, sheep, horses, cows, pigs etc.) as well as primates including humans. The factor may be obtained by isolation of endogenously produced factor in an appropriate cell line, or by recombinant expression.

As indicated, the cytokine of the invention is believed to inhibit differentiation by a mechanism which is distinct from gp130 and this feature may be used as a further characterising feature thereof.

Additionally its capacity to inhibit differentiation of ES cells cannot be eliminated by neutralising anti-DIA/LIF antiserum, nor by neutralising anti-IL-6 soluble receptor antiserum, nor by neutralising anti-CNTF antiserum.

The novel cytokine of the invention was discovered by generating cell lines that were DIA/LIF deficient and identifying in supernatants from such cell lines an activity that inhibited differentiation of ES cells. The supernatants from these cells in crude or partially purified form also form part of the present invention. Thus the present invention further provides an at least partially purified composition obtainable from supernatants of DIA/LIF deficient cells and comprising at least one polypeptide having the following characteristics

- the capacity to inhibit differentiation of ES cells (i) in the absence of DIA/LIF and (ii) in the absence of cytokines which act through gp130 and (iii) in the absence of interaction with gp130.

The capacity to inhibit differentiation of ES cells (iv) in the absence of interaction with LIF-receptor may further be used to characterise the partially

purified compositions. These may optionally further be characterised by features selected from the following:

- being distinguishable from DIA/LIF
- being distinguishable from IL-6/sIL-6R
- being distinguishable from CNTF
- being distinguishable from oncostatin M
- being distinguishable from cardiotrophin-1

taken individually or in combination.

At least partially purified polypeptide components of said compositions also fall within the present invention.

The invention may alternatively be defined as a cytokine designated ESRF which is obtainable by culturing cell line D7A3-PE and is characterised by

- the capacity to inhibit differentiation of ES cells
- being distinguishable from DIA/LIF
- being distinguishable from IL-6/sIL-6R
- being distinguishable from CNTF
- being distinguishable from oncostatin M
- being distinguishable from cardiotrophin-1

The invention further provides a method of producing the novel cytokine ESRF which comprises culturing a ESRF-producing cell line and isolating ESRF from the supernatant therefrom. Suitably the ESRF-producing cell line comprises the cell line D7A3-PE or a cell derived therefrom. The deposited cell line D7A3-PE constitutes a further aspect of the present invention.

The invention further provides culture procedures which utilise ESRF. One is a method of propagating ES cells which comprises propagating the cells in the presence of the cytokine designated ESRF. Another is a method of establishing ES cells which comprises culturing early embryos in the presence of the cytokine designated ESRF. These procedures can utilise ESRF as the sole ES cell-

propagation enhancing agent, or others may be included in the culture medium, for example DIA/LIF or other cytokines including ones which act through gp130. Propagation and/or establishment of ES cells in accordance with the invention may be monitored by culturing cells which include a selectable marker such as Oct-4*ßgeo*. Further the method of the invention may be utilised to promote the propagation of somatic stem cells such as haematopoietic stem cells.

In a particular embodiment of the invention, the cytokine of the invention, in combination with a second cytokine known to act via gp130, is used to derive, propagate and/or maintain a culture of embryonic stem cells in an undifferentiated state. For example, a combination of the cytokine of the invention with LIF is suitable for deriving, propagating and/or maintaining embryonic stem cells of rats or other species.

In this procedure for generating ES cells it is advantageous to culture embryos, which preferably are zona-free, on a feeder layer of cells which express matrix-associated LIF.

Suitable feeder layer cells are derived from fibroblasts. Examples include mouse embryo fibroblasts that have been transfected so as to express matrix-associated LIF. A suitable stably transfected cell line is designated DIA-M and has been deposited at the European Collection of Animal Cell Cultures (ECACC) on 31 May 1996 under Accession No. 96053101.

DIA-M was produced as a clonal derivative of C3H10T1/2 mouse embryo fibroblasts stably transfected with an expression vector containing a cDNA for the matrix-associated form of mouse LIF (Rathjen *et al.*, 1990) and an IRES-linked *neo* selectable marker. (See also WO 94/24301 for details of the expression system).

Thus in its more specific aspects there is provided a new methodology for isolating and propagating embryonic stem cells from other species. The approach is based on a novel combination of soluble LIF plus the cytokine Embryonic Stem

Cell Renewal Factor (ESRF) plus a feeder layer of genetically modified fibroblasts which overexpress the matrix-associated isoform of LIF (Rathjen *et al.*, 1990).

Using this protocol rat embryonic stem cells can be propagated indefinitely whilst retaining a euploid rat karyotype and characteristic stem cell morphology and marker expression. Furthermore a novel method for freezing and thawing the ES cells is described and procedures are given for chimaera production.

According to a further aspect of the invention there is provided a method for assaying and/or detecting growth factors that affect differentiation by a mechanism that is distinct from mechanisms involving DIA/LIF and/or gp130 interactions, which comprises culturing ES cells in the presence of a sample to be assayed, and detecting variation in growth or differentiation compared to cells cultured in the absence of the sample, characterised in that the ES cells have an LIF-negative (LIF(-)) and/or LIF receptor-negative (rLIF(-)) phenotype.

A specific embodiment of the invention is a cytokine having the following physicochemical properties:-

- apparent MW equal or greater than 100,000 Daltons in saline or 4M urea
- isoelectric point = 4.25-4.5
- stable above pH3, but inactivated below pH3
- inactivated by 0.5M NaOH
- sensitive to reduction by 10mM dithiothreitol, 30min, 37°C.
- inactivated by incubation with trypsin
- inactivated by incubation at 50°C
- stable to prolonged (6 months) storage at 4°C
- stable to freeze/thaw
- stable to exposure to 4M urea
- stable in 1% CHAPS or CHAPSO
- inactivated by 0.01% SDS

The invention further provides an established line of embryonic stem cells characterised by possessing at least five and preferably at least seven of the following features:

(i) the characteristic morphology of stem cells, including growth in clumps as small tightly packed cells with a high nuclear to cytoplasmic ratio,

(ii) expression of one or more specific markers selected from (a) alkaline phosphatase, (b) stage-specific embryonic antigen-1, (c) Oct-3/4,

(iii) non-expression of differentiation markers, for example H19 RNA,

(iv) substantial or unlimited propagation potential,

(v) stability to freezing and thawing,

(vi) stable euploid karyotype,

(vii) propagation dependent on cytokines,

(viii) in vitro differentiation inducible by withdrawal of cytokine(s), aggregation or chemical differentiation inducers,

(ix) ability to form teratocarcinomas comprising derivatives of endoderm, mesoderm and ectoderm,

(x) ability to colonise and/or reconstitute host tissues through the production of somatic stem cells and functionally differentiated progeny,

(xi) ability to colonise host embryos with contribution of functional differentiated progeny to chimeras,

(xii) ability to produce functional gametes in chimeras and generation of viable offspring,

(xiii) ability to integrate exogenous DNA,

and further characterised in that said cell line has a karyotype other than mouse. Such cells preferably possess at least five and preferably at least seven of the specified features (i) to (viii), and most preferably are further characterised by possessing at least one of the specified features (ix) to (xiii).

It will be appreciated that established lines of embryonic cells provided according to the invention need not necessarily exhibit each and every one of the features (i) to (xiii) listed above. Thus for certain species, one or more characteristics may be absent. For example in feature (ii) rodent embryonic stem cells may display all three of the specific markers mentionad above, i.e. (a) alkaline phosphatase, (b) stage-specific embryonic antigen-1 and (c) Oct-3/4,. However, stage-specific embryonic antigen-1 (feature (ii)(b)) may be absent or undetectable in embryonic stem cells of other species, especially large mammals such as primates.

Further, in respect of mammals having long gestation periods and/or reaching sexual maturity after a long period of time, it may not be practical to demonstrate the existence of features (x) and (xi). Also moral and legal constraints may make it impossible to demonstrate features (x) and (xi) in certain species such as human.

Description of Figures

The invention will now be described in more detail by way of example with particular reference to the accompanying drawings of which:

Figure 1 illustrates the response of *lif-r -/-* ES cells to cytokines

Figure 2 shows a phase contrast photomicrograph of rat ES cells

Figure 3 shows a metaphase chromosome spread from rat ES cells

Figures 4A & 4B show marker expression in rat ES cells of A. Alkaline phosphatase and B. Oct-4 immunostaining

Figures 5A, 5B & 5C show differentiation of rat ES cells into A. Trophoblast, B. Parietal endoderm and C. Bipolar cells

The production, isolation and characterisation of ESRF in accordance with the invention will now be described in more detail by way of example.

EXAMPLE 1

1.1 Generation of DIA/LIF-deficient ES cell lines

In order to abolish DIA/LIF function in ES cells, both alleles of the gene were deleted via two rounds of homologous recombination. ES cells in which one copy of the gene is inactivated were generated using a replacement vector in which the entire coding sequence of the DIA/LIF gene is replaced by a selectable HPRT mini-gene. After transfection into HPRT-deficient E14TG2a cells, HAT-resistant ES cell clones which had undergone the recombination event were identified by DNA hybridisation analysis using a probe external to the 3' arm of homology. To verify the expected replacement event, DNAs of positive clones were restricted with the appropriate restriction enzyme and hybridized to a 5' external probe. In total, 11 correctly targeted clones were identified from a primary screen of 89 colonies.

One germline competent clone, D6, was used for the deletion of the second allele. A hygromycin resistance cassette was substituted for the HPRT marker in the targeting vector. In parental cells this construct gave rise to homologous replacement events at a comparable frequency (10%) to that obtained with the HPRT vector. This second construct was electroporated into the D6 clone and transfectants were isolated by selection in hygromycin B. Three classes of homologous recombinant were identified amongst 162 colonies screened: recombination into the previously targeted allele had occurred in 30% of clones; integration into the 5' or 3' homology region of the wild-type allele was detected in 4 cases; and only three clones had undergone replacement of the wild-type allele. Deletion of DIA/LIF coding sequences was confirmed for these three clones by the absence of hybridisation with a full-length cDNA probe whereas clones which had undergone recombination into only one homology arm retained the gene. The loss of DIA/LIF mRNAs was confirmed using a highly sensitive ribonuclease protection assay. E14TG2a ES cells express relatively high level of both matrix-associated and diffusible forms of DIA/LIF mRNAs after induction of differentiation, as previously described for other ES cell lines. In contrast, no protected fragment could be detected in RNA preparations from undifferentiated or differentiated

double knock-out ES cells. These experiments confirm unambiguously that the DIA/LIF deletion is a null mutation. The similar phenotype of two independent clones, named D1C2 and D7A3, which were isolated from separate plates during the drug selection is reported in the following experiments.

1.2 Capacity of homozygous DIA/LIF-negative ES cells for differentiation *in vivo*

DIA/LIF-deficient ES cell clones were used to generate chimaeras. DIC2 and D7A3 ES cells were injected into C57BL/6 blastocysts and chimaeric offspring were produced. Chimaeras showed high ES cell contribution as judged by the sandy coat colour. This was confirmed by the determination of the degree of ES cell contribution to blood and tail of five chimaeric mice by glucose phosphate isomerase isozyme analysis. Both clones contributed to gametogenesis as indicated by the production of viable germ-line offspring. These results establish that the two rounds of selection applied to generate double knock-out ES cell lines have not abolished their normal developmental potential.

1.3 Stem cell renewal can occur in the absence of DIA/LIF

The differentiation of ES cells in high density monolayer culture does not result in the complete elimination of stem cells. We have previously proposed a model of feedback regulation of stem cell renewal in which the synthesis of DIA/LIF by newly differentiated ES cells contributes to the rescue of residual undifferentiated stem cells. The validity of this hypothesis was examined by investigation of the capacity of DIA/LIF-deficient ES cells for stem cell rescue following induction of differentiation. Wild-type, heterozygous and homozygous mutant ES cells were induced to differentiate at high cell density by exposure to 3-methoxybenzamide (MBA) for three days. After a further four days is basal medium, the number of undifferentiated ES colonies was determined by both morphological inspection and alkaline phosphatase staining. A similar number of undifferentiated ES colonies were recovered from the wild-type and two heterozygous ES cell cultures. In contrast, the number of ES colonies was around 3-fold lower for the two DIA/LIF-negative ES clones. This decrease confirms that

the autocrine and paracrine production of DIA/LIF is a major component in the feed back regulation of stem cell renewal in differentiating ES cell cultures. Significantly, however, stem cell renewal was not completely abolished in the absence of DIA/LIF, suggesting that another regulatory factor is operative in this system.

1.4 Inhibition of ES cell differentiation by a soluble factor (ESRF)

In order to preclude the possibility that the culture conditions imposed a selection for rare differentiation-defective variants, the stem cell rescue capacity of DIA/LIF-deficient ES cells was further investigated in co-culture assays. Convenient indicator cell lines were generated by integration of a ß-galactosidase reporter gene into the Oct-4 locus of DIA/LIF-negative ES cells by homologous recombination. The expression of ß-galactosidase in such targeted clones is restricted to undifferentiated stem cells. The indicator cells were plated on layers of MBA-induced differentiated wild-type or mutant clones. After 4 days, the number of undifferentiated ES cells colonies derived from the indicator population was determined by staining for ß-galactosidase activity. Once again, the results indicate that the capacity of DIA/LIF-deficient differentiated cells to inhibit ES cell differentiation is reduced relative to the parental cell line, but is not abolished. Similar results were obtained using retinoid-induced differentiated cells as the feeder layer.

To determine whether the effect was due to a diffusible factor, a second kind of co-culture experiment was carried out in which the indicator cells were plated on a microporous insert above the layer of differentiated cells. The insert membrane prevents cell-cell contact between the two cell populations but allows the access of diffusible factors. Both parental and Oct-4-tagged ES cells were used as indicators, staining for alkaline phosphatase and ß-galactosidase respectively. Similar results were obtained in both cases. The greater activity in wild-type cultures is significantly reduced in the presence of neutralizing DIA/LIF antiserum, but is not eliminated. This indicates that wild-type cells also synthesize active factors other than DIA/LIF. The residual activity in the presence of anti-DIA/LIF

was similar to that produced by DIA/LIF-negative cells which suggests that expression of the responsible factor is not significantly up-regulated in these cells.

These data establish that a soluble factor or soluble factors other than DIA/LIF which is able to prevent ES cell differentiation is synthesized by both wild-type and DIA/LIF-deficient differentiated cells. However, the decreased numbers of colonies generated in response to the mutant cells or to wild-type cells in the presence of neutralising anti-DIA/LIF indicate that this factor is produced in limiting amounts.

1.5 Isolation of a differentiated cell line expressing high levels of a stem cell renewal factor (ESRF)

In order to facilitate characterization of the stem cell renewal activity, we established differentiated cell lines from embryoid body outgrowths of the DIA/LIF-negative ES cells. Several of these cell lines produced levels of activity which were readily detectable in their culture supernatants. One cell line, named D7A3-PE, could be propagated indefinitely and produced high levels of a soluble factor which could maintain undifferentiated ES cells. In addition to their characteristic morphology, ES cells cultured in D7A3-PE conditioned medium continued to express markers of the undifferentiated state such as alkaline phosphatase and Rex-1 mRNA and in the case of Oct-4 tagged cells retained ß-galactosidase activity. This conditioned media was effective on several independent ES cell lines. ES cells cultured in the presence of conditioned media or partially purified ESRF formed tight, rounded up colonies, morphologically distinguishable from both differentiated cells and ES cells maintained in DIA/LIF. ES cells serially passaged in the presence of ESRF and retained the capacity for multilineage differentiation. The active factor could be concentrated at least 20-fold by ultrafiltration and was destroyed by incubation with trypsin, consistent with a proteinaceous macromolecule. The factor was inactivated by heating to 50°C or by acidification below pH3.

1.6 Purification and biochemical properties

For purification of ESRF, conditioned medium is prepared from D7A3-PE cells in the absence of serum. Cells are inoculated into 150cm^2 tissue culture flasks in normal growth media and grown to near confluence. Cultures are then rinsed with PBS and transferred into defined medium consisting of a 50:50 mix of GMEM and Ham's F12 basal media supplemented with 100μM 2-mercaptoethanol, 1μg/ml insulin, 5μg/ml transferrin and 10nM sodium selenite. Cultures are incubated for 4 hours then the medium is discarded and replaced with fresh defined medium (50ml/flask). This medium is conditioned by incubation with the D7A3-PE cells at 37°C for 96 hours. The medium is then harvested and replaced. Three successive harvests can be obtained from each culture. The harvested medium is clarified by centrifugation and passed through a sterile 0.2μm filter. The ability to inhibit ES cell differentiation is routinely detectable at a 1 in 10 dilution of the unfractionated conditioned medium though full activity over a four day assay requires no greater than 25% dilution. Activity in the conditioned media is stable upon freezing and thawing, but is lost upon incubation at 50°C for 30 min, acidification to pH2 or incubation with trypsin. Activity can be concentrated by ultrafiltration in an Amicon cell using a membrane with a nominal molecular weight cut-off of 100,000 Daltons. Quantitative recovery of biological activity is obtained, indicating that native ESRF has a molecular weight in excess of 100,000 Daltons.

ESRF is quantitatively recovered from conditioned medium by stepwise precipitation with saturated ammonium sulphate. ESRF activity is recovered in the 25-35% fraction. This fraction contains 20-25% of total protein. Upon reconstitution in 1/100 volume of starting medium, a viscous solution is obtained which partitions into fluid and gel phases upon cold storage. Both phases contain biologically active ESRF. The gel material is likely to consist of highly glycosylated extracellular matrix components. ESRF can be extracted from the gel by incubation with salt buffers or more efficiently with 0.1% CHAPS. This finding suggests that ESRF has avidity for ECM components. Consistent with this, significant levels of ESRF activity can be extracted directly from monolayers of D7A3-PE cells by incubation for 2 hours with basal medium containing 0.1% CHAPS. (Under such

conditions the cells remain viable with no evidence of significant lysis). It is possible that ESRF may exist in isoforms which associate differentially with extracellular matrix. Alternative protocols for obtaining an ESRF-containing fraction by ammonium sulphate precipitation are given in Appendix 9.

Ultracentrifugation studies (see appendix 10) suggest that ESRF activity is not significantly associated with plasma membranes or subcellular organelles but is a soluble protein or protein complex.

The maximum concentration required for biological activity is 1.5nM, based on the protein content of urea resolubilised ammonium sulphate precipitated material (see Appendix 9) and assuming a molecular weight equal to 100,000 Daltons.

Further fractionation of ESRF may be achieved on lectin columns. ESRF binds to soya bean lectin and to lentil lectin, but not to wheat germ agglutinin (appendix 11). Bioactivity is not affected by the addition of heparin to the assay media.

Upon size exclusion chromatography of the ammonium sulphate fraction using TSK G3000SWXL HPLC column in 50mM sodium phosphate buffer, pH7.2, ESRF migrated as a single peak with a retention time shorter than BSA, indicative of a molecular weight of 100-150,000 Daltons.

ESRF can be further purified by solution phase electrofocussing of the ammonium sulphate fractionated material. Focussing may be performed in the presence of 1% CHAPS and 2% ampholytes in a BioRad Rotofor cell. ESRF is recovered quantitatively in 2-4 fractions of pH 4.25-4.5. ESRF is insoluble in these fractions, but resolubilizes upon direct addition to culture medium for the ES cell bioassay or upon adjustment of buffer pH. SDS gel electrophoresis shows that two major proteins with apparent molecular weights of 115,000 and 180,000 Daltons are specific to the active fractions.

Further purification of ESRF may be achieved by heparin agarose affinity chromatography. The sample is loaded in 20 mM phosphate buffer, pH3.5, containing 0.1% CHAPS. Under these conditions most material does not bind to the column and a significant purification of ESRF can be obtained. ESRF is not eluted with 100mM salt, but elutes over a broad range of higher salt concentrations. The heterogenous elution profile is suggestive of variable glycosylation. (Similarly heterogenous elution profiles are also obtained on anion exchange chromatography). Bioactive fractions contain very low levels of protein (undetectable at 280nm). Protein is also undetectable by either coomassie blue staining, conventional silver staining, or silver staining combined with periodic acid preincubation (for improved detection of glycoproteins) of SDS PAGE gels loaded with up to 10-fold higher amounts of material than required for full biological activity. The low amount of protein present indicates that ESRF is fully active at nanomolar concentrations.

Biotinylation of pooled fractions of active material followed by SDS PAGE in the presence of reducing agent and immunoblotting revealed the presence of a heterogeneous doublet of apparent molecular weight 70-80,000. No other bands were apparent. This observation confirms that these fractions are highly purified. It further indicates that native ESRF may be a disulphide-linked dimer.

The concentration of IL-6 in the conditioned medium was determined using the highly sensitive B9 cell proliferation assay. Conditioned medium did induce proliferation of B9 cells but the mitogenic response 'equated to a concentration of less than 5 pg/ml IL-6. That this activity was due to IL-6 was confirmed by the use of a neutralizing IL-6 soluble receptor antibody, RS13. The bioactivity was inhibited totally in the presence of RS13. In contrast, inhibition of ES cell differentiation by D7A3-PE cell conditioned medium was not modified by the addition of RS13, indicating that this low level of IL-6 is not responsible for the effect of this media. This conclusion was further substantiated by the finding that the conditioned medium was not mitogenic for BAF-mgp130 cells. The latter are responsive to IL-6 only in the presence of soluble IL-6 receptor, so the negative

result in this assay indicates that the D7A3-PE cell conditioned media does not contain any IL-6 soluble receptor.

The potential involvement of CNTF was examined both by specific immunodepletion of conditioned medium with the 4-68 anti-CNTF mAb, and by the use of a neutralizing anti-CNTF antiserum. Neither treatment affected the ability of D7A3-PE cell conditioned medium to inhibit ES cell differentiation. Furthermore, activity was retained on addition to RS13 anti-IL-6R antiserum to medium previously depleted with anti-CNTF.

These results establish that neither IL-6/sIL-6R nor CNTF are responsible for the inhibition of ES cell differentiation by differentiated DIA/LIF-deficient ES cells.

1.7 The self-renewal factor is distinct from mouse oncostatin M and from cardiotrophin-1

Mouse cardiotrophin-1 has a molecular weight of 22,000 Daltons. Mouse oncostatin M has a molecular weight of 30-40,000 Daltons. ESRF, in contrast, has an apparent molecular weight in excess of 100,000 Daltons. It is quantitatively retained by ultrafiltration membranes with a cut-off of 100,000 Daltons even in the presence of 4M urea and has a mobility on size exclusion chromatography corresponding to a molecular weight greater than 100,000 Daltons.

1.8 ESRF is active on LIF-receptor deficient ES cells

LIF and related cytokines act through a heterodimeric receptor consisting of the low affinity LIF receptor and gp130. ES cells lacking LIF receptor were generated by two rounds of gene targeting. These cells can be propagated using the combination of IL-6 and soluble IL-6 receptor which acts via gp130 homodimers without involvement of LIF-receptor. LIF-receptor negative ES cells are pluripotent and contribute to multiple tissues in chimaeras. They show no self-renewal response to LIF, CNTF, oncostatin M or cardiotrophin-1. As shown in Figure 1, however, LIF-receptor deficient ES cells remain responsive to ESRF. This finding confirms that ESRF is distinct from the LIF group of cytokines.

1.9 The self-renewal factor (ESRF) does not act through direct interaction with gp130

All the cytokines described to date which are capable of sustaining ES cell self-renewal act through receptor complexes containing the gp130 signal transducer. Monoclonal antibodies have been raised against mouse gp130 which are capable of blocking gp130-mediated signal transduction (Saito et al., unpublished data). In the presence of these antibodies, ES cell responses to DIA/LIF, IL-6/sIL-6R, CNTF and human OSM are inhibited and the cells differentiate (Saito et al, unpublished data). The activity of WEHI-3B cell conditioned medium (a source of mouse cardiotrophin-1) on ES cells also is completely abolished by anti mouse gp130.

The effect of two different neutralising anti-mouse gp130 antibodies on the maintenance of ES cell self-renewal by ESRF was investigated. In the presence of ESRF, neither antibody inhibited the production either of alkaline phosphatase-positive undifferentiated colonies by wild-type ES cells, or of ß-galactosidase-positive, G418-resistant colonies by Oct-4-targeted cells (Mountford *et al.*, 1994). The integrity of the antibodies at the end of the assay was confirmed by retention of the ability to block mitogenic stimulation of BAF-mgp130 cells by IL-6/sIL-6R.

The results of these experiments with neutralising antibodies against mouse gp130 indicate that the effect of ESRF on ES cell maintenance is not mediated via direct activation of gp130, in contrast to the actions of all previously described ES cell self-renewal factors.

Addition of cytokines which act via gp130 to ES cells results in activation of STAT3 and induction of the immediate early gene *tis11*. Under the same conditions, however, ESRF administration causes no apparent increase in STAT3 activity as determined by gel shift analysis and no induction of *tis11* transcripts by

RNase protection assay. This indicates that ESRF action is mediated via a distinct intracellular signalling pathway(s) from gp130 signal transduction.

The biological response of ES cells to ESRF is also distinguishable from their response to LIF or related cytokines. The ES cells are characteristically more compact in the presence of ESRF and form very tight, rounded, colonies which have a tendency to detach from the culture surface. On transfer to LIF-containing medium these colonies adopt a slightly more flattened and spread appearance, typical of ES cells cultured in LIF. Undifferentiated colonies can be maintained for at least 11 days using ESRF alone. However, the colony size does not appear to increase after the first 4 days, in contrast to the effect of LIF which promotes continuous stem cell expansion. Additive or synergistic effects on stem cell propagation are apparent on combination of sub-optimal amounts of LIF and ESRF. These data are consistent with distinctive effects on the self-renewal process.

1.10 Mouse ES cells maintained using ESRF remain pluripotential

ESRF sustains the undifferentiated phenotype of ES cells *in vitro*. This effect persists for at least 11 days. However, proliferation is limited and cultures cease to expand after about four days. This contrasts with cultures propagated in the presence of LIF which undergo a continuous increase in stem cell numbers. To determine whether ES cells maintained in ESRF are capable of contributing to chimaeras, cells were cultured at clonal density in the presence of ESRF for four days. They were then transferred to LIF-containing medium to facilitate expansion of the stem cells prior to blastocyst injection. This protocol was applied to the ES cell line Zin40 which carries a constitutively expressed nuclear localised ß-galactosidase marker.

Zin40 cells plated in control medium underwent complete differentiation and did not give rise to any ES cell colonies upon replating in the presence of LIF. Cells plated in ESRF, however, remained undifferentiated as described above and engendered many alkaline phosphatase positive stem cell colonies on replating. Cells from replated and expanded cultures contributed extensively to chimaeras as

determined by the widespread presence of ß-galactosidase positive cells in mid-gestation embryos. These chimaeric embryos were morphologically normal. The levels of Zin40 contribution were comparable to those obtained from parallel injections of cells maintained in LIF only.

1.11 Uses of ESRF

Regulation of the balance between stem cell renewal and differentiation is crucial for tissue diversification during embryogenesis and for tissue renewal and repair in adult mammals. The generation of DIA/LIF-deficient ES cells allowed us to study the role of this cytokine in stem cell renewal and differentiation and to characterize other factors involved in the regulation of this process. The results establish that DIA/LIF-deficient differentiating cultures retain some capacity for maintaining a population of undifferentiated cells. The findings indicate that factors and signalling pathways other than those characterised to date have the capacity to maintain the self-renewal of pluripotential ES cells. This may prove of fundamental importance for the isolation and propagation of stem cells from other species.

The propagation and differentiation of murine pluripotential embryonic stem (ES) cells is controlled by specific cytokines. The proliferation of ES cells *in vitro* is sustained through the activation of intracellular processes associated with the signal transducer gp130. In an attempt to define the relative contributions of different cytokines to self-renewal in ES cell cultures we generated ES cells lacking a functional gene for the cytokine Differentiation Inhibiting Activity (DIA/LIF). These cells show a significantly reduced capacity for stem cell renewal when induced to differentiate, indicating that DIA/LIF is the major regulatory cytokine present in normal ES cell cultures. However, undifferentiated ES cell colonies are still produced in the complete absence of DIA/LIF. This is due to the secretion of a soluble, macromolecular, activity by differentiated ES cell progeny. In addition to DIA/LIF, the cytokines ciliary neurotrophic factor (CNTF), interleukin-6 in combination with soluble interleukin-6 receptor (IL-6/sIL-6R), cardiotrophin-1 and oncostatin M can each activate gp130 and support ES cell propagation. The

involvement of either CNTF or IL-6/sIL-6R in our system has been precluded through the use of neutralising antisera against these factors. Most significantly, the effect of all the aforementioned cytokines on ES cells is abolished in the presence of neutralising antibodies against mouse gp130, whilst the activity in D7A3-PE conditioned media is unaffected. These findings establish that ES cell self-renewal can be sustained via a gp130-independent signalling pathway and that differentiated ES cell derivatives secrete a factor which activates this pathway.

The cell line designated D7A3-PE has been deposited at the European Collection of Animal Cell Cultures (ECCAC) on 18th November 1994 under Accession No 94111845.

EXAMPLE 2 - Derivation of Non-Mouse ES Cells

There has only been a single report to date of establishment of purported ES-like cells from the rat (Iannacone *et al* [1994] Dev. Biol.) despite intense effort in several laboratories. However, as indicated above, Iannacone's cells were later shown to have a mouse and not a rat karyotype. The potential application of ESRF to derivation of rat ES cells has therefore been investigated.

2.1 Derivation and propagation of rat embryonic stem cells in the presence of ESRF

Rat blastocysts were placed in culture on irradiated feeder layers of C3H10T1/2 derived feeder cells in standard ES cell culture medium containing LIF plus ESRF. Inner cell mass clumps were picked, dissociated in trypsin and replated in identical conditions. Colonies of small, morphologically undifferentiated cells which arose were picked individually, dissociated and replaced. In this way stem cell cultures have reproducibly been initiated from approximately 50% of rat blastocysts of Fischer and Sprague Dawley, and at a lower percentage of BDIX and DA strains. The undifferentiated cells can be maintained for prolonged periods and expanded extensively, by regular passaging. They can be frozen by conventional procedures and recovered from storage in liquid nitrogen.

In more detail, the procedure was as follows:

2.2 Protocol for rat ES cell derivation

2.2.1 Female rats are paired with males and checked every morning for the presence of sperm in the vagina. On the morning of the 5th day of pregnancy (day on which sperm found = day 1) blastocysts are flushed from the uteri, using medium 1 (Appendix 1). Zonae pellucidae, if present, are removed by brief exposure to acid Tyrode's medium.

2.2.2 Feeder layers are prepared from DIA-M fibroblasts. DIA-M cells are a clonal derivative of C3H10T1/2 mouse embryo fibroblasts stably transfected with an expression vector containing a cDNA for the matrix-associated form of mouse LIF (Rathjen *et al.*, 1990) and an IRES-linked *neo* selectable marker. These cells express high levels of recombinant matrix LIF. Feeder layers are made by dispensing approximately 75,000 gamma-irradiated DIA-M cells to a 15 mm diameter well of a 4-well tissue-culture plate (from Nunclon). 0.5 ml medium 2 (Appendix 1) is added to each well.

2.2.3. The zona-free embryos are cultured in these wells for 5 days. After the embryos attach to the feeders (after 2-3 days) the medium (medium 2) is changed daily.

2.2.4. After 4 days in culture, the large masses of cells are picked individually from the feeder layers, roughly broken up by trituration in fine Pasteur pipettes, and transferred individually to wells containing DIA-M feeder layers (prepared as above) and 0.5 ml medium 3 (Appendix 1) per well.

Dishes are incubated at 37°C, 7%CO_2 in air, and the medium is changed daily. These culture conditions are used in all further steps.

2.2.5. Small colonies of small, clear, morphologically undifferentiated cells can be seen in the wells after 1-7 days in culture. When large enough, these

are picked for trypsinisation or breaking up. (See step 5). If too small for trypsinisation, colonies are transferred intect to fresh feeders. Alternatively, if the well does not contain too much differentiating tissue, colonies may be removed from the monolayer and allowed to reattach.

2.2.6. Colonies to be trypsinised are rinsed briefly in 3 changes of phosphate-buffered saline and incubated in .05% trypsin with 0.5 mM EDTA and 0.1% chicken serum for 45-60 seconds. They are then transferred to a drop of medium 3, disaggregated into single cells and small clumps by trituration, and added to a new well (as described in step 3 above). Alternatively colonies may be physically broken up into clumps without trypsin treatment by cutting them apart with glass needles and triturating the fragments with a finely drawn pipette.

2.2.7. Cell lines are maintained by repeated passaging of colonies as described above. As the number of colonies per well increases, cultures can be split into several wells and/or transferred to larger wells.

2.2.8. Cells are karyotyped at intervals (every 3-4 passages) to ensure that euploidy is maintained, according to the protocol detailed in Appendix 2.

2.2.9. Cells are examined at intervals (every 5-6 passages) for the expression of oct-4 protein (Appendix 3), oct-4 mRNA (Appendix 4) alkaline phosphatase (Appendix 5) and SSEA-1 (Appendix 6).

2.2.10. Cultures are maintained in the presence either of penicillin and streptomycin, or gentamycin. The type of antibiotic used is changed every 3 to 4 weeks to minimise the risk of antibiotic-resistant strains of microorganisms developing.

2.2.11. To make chimaeric animals, cells are disaggregated in trypsin as described in step 5 above, and introduced into host embryos. Three examples of protocols for chimaera production are described below:

a) Host embryos at the early blastocyst stage are flushed from uteri of female rats in the morning of the 5th day of pregnancy (early blastocyst stage) using medium 1. They are cultured for 24 hours in medium 4, and the next morning disaggregated cells are injected into the blastocyst cavity. The embryos are allowed to recover in the incubator for two hours, and are then transferred to the uteri of rats in the 5th day of pseudopregnancy.

b) Host embryos at the early blastocyst stage are collected as in 2.2.11.a. They are injected immediately with disaggregated cells and transferred immediately to the uteri of rats in the 4th day of pseudopregnancy.

c) Host embryos are flushed from the oviducts of rats in the 4th day of pregnancy (8-cell stage) using medium 1. Using a micro-manipulator, the zonas are slit and several cells are introduced into the sub-zona space. Embryos are then returned to the uteri of females in the 5th day of pseudopregnancy.

2.2.12. Cells and host blastocysts are derived from strains with different coat colours, and chimaeric animals can be identified by colour about a week after birth. Alternatively, micro satellite markers can be used to distinguish donor cells and host derived populations.

2.2.13. Cells may be transfected by the procedure described in Appendix 7.

2.2.14. Cells may be frozen by the procedure described in Appendix 8.

2.3 Conclusions

Both LIF and ESRF are essential for maintenance of the undifferentiated cells. Upon withdrawal of either component the cultures differentiate completely into trophoblastic cells.

The ES cell status of the undifferentiated cells after extended culture periods is evidenced by one or more of: (i) maintenance of overt ES cell morphology; (ii) induction of overt differentiation by withdrawal of cytokines, (iii) expression of the early embryo and ES cell specific antigen SSEA-1, (iv) expression of the pluripotent cell specific transcription factor Oct-3/4, (v) expression of alkaline phosphatase; and (vi) non-expression of H19.

The characteristic stem cell morphology of rat ES cell cultures is shown in figure 2. these cells are morphologically indistinguishable from mouse ES cells cultured under the same conditions. The rat chromosome complement of the cultures is shown in Figure 3. Expression of markers is shown in Figure 4; upper panel alkaline phosphatase, lower panel Oct-4 immunostaining. Figure 5 shows examples of rat ES cell differentiation; a. trophoblast, b. parietal endoderm, c. unidentified bipolar cells.

These findings provide a direct demonstration that ESRF can collaborate with LIF (or other cytokines which act via gp130) to enable the establishment of ES cells.

2.4 Further Characterisation of Rat ES Cells

Using the protocol we give in section (2.2), we can reproducibly derive cell lines from the rat blastocyst, which are morphologically identical to mouse ES cells grown in the same conditions. The cells are small, with high nuclear to cytoplasmic ratio and grow tightly packed together in clumps with a characteristic rounded, smooth, transparent appearance. These colonies are morphologically indistinguishable from clumps of mouse ES cells in the same conditions. Although the majority of cells in these cultures maintain an undifferentiated phenotype, some

degree of differentiation occurs. Cells most frequently differentiate as parietal endoderm and giant cells or other trophoblast-like cells, but other cellular morphologies are occasionally seen.

Besides having the characteristic morphology of mouse ES cells, the rat cells express the same markers. Alkaline phosphatase is expressed by primordial germ cells in the rat and mouse, and by mouse ES cells. The rat cell lines we have derived are also strongly positive for alkaline phosphatase, but cease to express this enzyme when the cells differentiate. The rat cells stain positively with an antibody to SSEA-1 (stage-specific embryonic antigen 1) which is also expressed by primordial germ cells and mouse ES cells. Finally, immunohistochemical staining has demonstrated that oct-4 (a transcription factor expressed only by totipotent cells, such as primordial germ cells, the inner cell mass of the blastocyst, and mouse ES cells) is expressed by our rat cells.

We have maintained lines of cells with these morphological characteristics and which express oct-3/4, SSEA-1 and alkaline phosphatase for more than 25 passages in vitro (over 3 months).

We have confirmed that these are indeed rat cells by examining their karyotype. They have a modal chromosome number of 42 and possess metacentric and submetacentric chromosomes characteristic of rats and not found in normal mouse strains. The cell lines remain euploid at high passage numbers.

If cells are frozen according to the protocol in Appendix 8, colonies may be stored in liquid nitrogen and subsequently thawed and maintained in culture with no increase in incidence of differentiation, or alteration of ploidy.

The maintenance of an undifferentiated phenotype in these lines is dependent upon 3 factors, each of which should be present in the system if cell lines are to remain viable and undifferentiated for more than a few passages. The medium must contain soluble DIA/LIF and ESRF (see sections 2.1), and the cells

are preferably grown upon feeder layers of mitotically inactivated DIA-M cells (section 2.2.2). If either LIF or ESRF is removed from the systems, the cells will all eventually differentiate (mostly as trophoblast-like cells) or die (Table 1, below). Once established, rat cells can be propogated on alternative feeder layers or even on gelatin-coated plastic. Later passage cells may also be grown in the absence of ESRF.

	maximum passage number
with DIA/LIF, ESRF, and DIA-M feeders	>25
without DIA/LIF	1-2
without ESRF	4
without DIA/M feeders	4-5

Chimaeras may be produced from these cells by introduction into host embryos using the procedures given in section 2.2.11.

Genetically modified rats may be produced by transgene integration or by gene targetting in rat ES cells followed by Chimaera production and germline transmission.

2.5 Activity of ESRF on Human Teratocarcinoma Cells

The effects of conditioned medium and ammonium sulphate fractionated ESRF on the multipotent human teratocarcinoma cell line GCT 27X1 were examined. The assay involves growth of cell colonies from single cells in the absence of a feeder layer in serum-containing medium over a 10-14 day period. Cells are observed on a daily basis using phase contrast microscopy. Under the conditions of the assay, cell survival and colony formation in control medium is low. ESRF preparations consistently demonstrated a positive effect on stem cell survival in these assays. Cell viability was enhanced in the early part of the assay and final colony number was increased substantially. Thus ESRF or an activity

associated with it is active in promoting *in vitro* survival of human pluripotent teratocarcinoma stem cells.

APPENDIX 1: Media

Medium 1: Flushing medium. PB1 (see Whittingham, D., 1971. Culture of mouse ova. J Reprod Fert (Suppl. 14):7-21) with 10% fetal calf serum and antibiotics.

Medium 2: ESRF medium. 80% GMEM, 20% fetal calf serum, 2000 units human DIA/ml, and either 50 units/ml penicillin, 50 mg/ml streptomycin, or 50 mg/ml gentamycin. Semi-purified ESRF is added at a concentration determined by bioassay of mouse ES cells (see Appendix 9).

Medium 3: Gardners G1 or G2 (Barnes *et al.*, 1995, Human Reproduction 10:3243-3247).

APPENDIX 2: Karyotyping of cells

1. Colonies of cells to be karyotyped are picked from their dishes and cultured in Colcemid at 0.8 mg/ml for 2 hours in medium 3, in dishes containing feeder layers of DIA-M cells.

2. Cell clumps are removed and rinsed briefly in changes of PBS (Dulbecco's phosphate-buffered saline).

3. Clumps are rinsed briefly in hypotonic saline (1% Na citrate, no older than 1 week), transferred to fresh saline and left for 8-10 minutes.

4. Clumps are transferred to a solid watchglass and excess hypotonic saline removed.

5. The dish is then filled with freshly made up 3:1 fixative (3 parts absolute ethanol to 1 part glacial acetic acid) and left for 1 hour.

6. Clumps of cells are transferred to a clean microscope slide and watched under a microscope until the fixative has almost entirely evaporated.

7. 2-4 drops of 60% acetic acid are then added to the cells, causing the tissue to disaggregate.

8. The drop is kept moving around the slide by blowing until it is completely evaporated.

9. Slides are stained in 2% Giemsa stain in Giemsa buffer (from Gurr). They may be mounted, or can be examined unmounted.

APPENDIX 3: Immunohistochemical staining for oct-4 protein

1. Medium is removed from wells and cells are washed twice with complete PBS (Dulbecco's phosphate-buffered saline with 1.5 mM MgCl2 and 1 mM CaCl2).

2. Cells are fixed for 10 minutes in 3.7 formalin in complete PBS.

3. Cells are permeabilised for 15 minutes with 0.2% Triton in complete PBS.

4. Cells are blocked for 20 minutes in goat serum at the dilution suggested in the protocol of the Vectastain Elite ABC kit, supplied by Vector Laboratories.

5. Primary antibody (affinity-purified anti-oct-4 antiserum from rabbits Palmieri *et al.*, 1995) is diluted 1:5000 in PBS and added to the cells for 30 minutes.

6. Cells are washed in PBS for 10 minutes.

7. The biotinylated secondary antibody (goat anti-rabbit) is prepared according to the protocol of the Vectastain kit (Vector Laboratories) and added to the cells for 30 minutes.

8. Cells are washed in PBS for 10 minutes.

9. The "ABC reagent" is prepared according to the protocol of the Vectastain kit (Vector Laboratories), allowed to stand for 30 minutes, and added to the cells for 30 minutes.

10. Cells are washed in PBS for 10 minutes.

11. Peroxidase substrate is prepared from the "VIP kit" supplied by Vector Laboratories according to instructions.

12. Peroxidase substrate is added to the cells fro 2-10 minutes, until a suitable intensity of color develops.

13. Cells are washed for 5 minutes in tap water.

14. Cells should be examined and photographed at once.

APPENDIX 4: in situ staining for oct-4 mRNA

Note: all solutions are made with DEPC-treated water

1. The probe is synthesized from a Stu 1 fragment of a Bluescript plasmid carrying the POU homeodomain of the oct-4 gene, with 1.75 mM digoxigenin-11-UTP in the synthesis mixture .

2. Cells attached to tissue-culture plates are fixed in 4% paraformaldehyde in PBS (Dulbecco's phosphate-buffered saline) at 4°C overnight.

3. They are rinsed with PBT (PBS + 01.% Tween) and dehydrated in a graded series of methanol (25%, 50%, 75% and 100%) and stored at -20°C until needed.

4. Cells are rehydrated through the methanol series, rinsed with PBT, and washed 3 times in RIPA (1% NP-40, 0.5% NaDOC, 0.1% SDS, 1 mM EDTA and 50 mM Tris in 150 mM NaCl).

5. Cells are first washed in 1:1 hybridisation buffer:PBT (hybridisation buffer:50% formamide in 10xSSC pH 4.5, with 0.05% of a stock solution of 100 mg/ml heparin, and 0.1% Tween 20), and then with 100% hybridisation buffer.

6. Hybridisation buffer with herring sperm DNA (100 μg/ml) and yeast transfer RNA (10 mg/ml) is added to the cells, and the dishes are incubated at 70°C overnight.

7. The probe is denatured, added to the wells at a dilution of 1:100 - 1:200, and the dishes incubated at 70°C overnight.

8. Cells are washed briefly at 65°C with posthybridisation wash buffer (50% formamide in 2xSSC with 0.1% Tween 20). This wash is followed by 3 further washes at 65°C of 20 minutes each.

9. Cells are allowed to cool to room temperature and washed 3 times with TBST (0.8% NaCl, 0.02% KCl, 2.5% 1M Tris-HCl pH 7.5).

10. Sheep serum is inactivated by heating to 30 minutes at 70°C with regular shaking. 10% inactivated sheep serum in TBST is added to the cells and the plates are left at room temperature for 1 hour.

11. The serum is removed and replaced with anti-digoxigenin alkaline phosphatase conjugated Fab fragments in 1% sheep serum diluted in TBST to a concentration of 1:2000. Dishes are stored at 4°C overnight.

APPENDIX 4 (contd.) : in situ staining for oct-4 mRNA

12. Cells are washed briefly 3 times with TBST at room temperature, and then a further three times for at least 2 hours total time.

13. Cells are washed 3 times in alkaline phosphatase buffer (100 mM NaCl, 50 mM MgCl2, 0.1% Tween 20, 100 mM Tris pH9).

14. The wash is removed and cells stained in the dark in a solution of 4.5 ul NBT and 3.5 ul BCIP (x-phosphate) per ml alkaline phosphatase buffer.

15. When a suitable intensity of stain is observed, the reaction is stopped by rinsing 3 times in PBT with 1 mM EDTA. Cells can be stored for a short time in this solution at 4°C.

APPENDIX 5: Alkaline phosphatase staining of cultured cells

1. Cells growing in tissue culture dishes are fixed in cold 80% ethanol for at least one hour. They may be stored at 20°C in this fix for several weeks.

2. Cells are hydrated by washing for 20 minutes each in solutions of 50% absolute ethanol, 30% absolute ethanol, and two changes of distilled water.

3. Cells are stained in 0.05% Fast Red TR Salt, 0.01% a-naphthyl phosphate. 0.03% MgCl2, and 0.22% borax for 5-10 minutes.

4. Cells are washed in distilled water and observed immediately. If necessary, cells can be stored in 50% glycerine in water.

APPENDIX 6: Immunohistochemical staining for SSEA-1

1. Cells growing on feeder layers attached to glass coverslips are placed in wells for ease of handling. They are washed twice in PBS (Dulbecco's phosphate-buffered saline).

2. Primary antibody (monoclonal anti-SSEA-1, obtained from J. Ansell) is diluted 1:1000 in serum-free medium with 0.15% BSA and buffered with Hepes. This is added to the cells and the dishes incubated at 4°C for 45 minutes.

3. Cells are washed 3 times in cold medium.

4. The secondary antibody (anti-mouse IgM, FITC labelled) is diluted 1:10 with medium, added to the cells for 30 minutes at 4°C.

5. Cells are washed 3 times in cold medium.

6. Cells are washed 3 times in cold PBS.

7. Cells are fixed in cold 95% methanol/5% acetic acid for 3 minutes.

8. Cells are rinsed in PBS, the coverslips removed and inverted on drops of 10% PBS:90% glycerin on glass slides.

9. Slides are examined under a fluorescence microscope.

APPENDIX 7: Transfection of cells

Procedure 1: Calcium phosphate co-precipitation.

1. Cells are trypsinised and transferred to fresh wells containing feeder layers and medium as usual.

2. Dishes are placed in an incubator at 37°C with 2.5% CO2.

3. Transfection mix (to a final concentration of 50 ul/ml BES, 0.012 M CaCl2 and 1 ug/ml DNA) is added to wells and the cells incubated overnight.

4. The transfection mix is removed next morning and replaced with normal medium.

5. Transfected cells are selected and/or identified according to the method determined by the incorporated DNA.

Procedure 2: Lipofection

1. Make up transfection solution, using lipids from the PerFect transfection kit from invitrogen, and Optimem from Gibco.

    a. add lipid to Optimem (12 μl/ml)
    b. add DNA to Optimem (2 μl/ml)
    c. mix lipid/Optimem with DNA/Optimem
    d. Incubate at 37°C for approximately 15 minutes.

2. Wash colonies of cells in three changes of PBS.

3. Transfer colonies to trypsin for 1 minute.

4. Transfer colonies to a drop of Optimem and triturate with a pulled Pasteur pipette.

5. Remove medium from wells with feeder layers and replace with Optimem plus lipid and DNA.

6. Add trypsinised cells to the well and incubate for 5 hours.

7. Remove Optimem and replace with cell medium (medium 2).

Procedure 3: Electroporation

1. Wash colonies 3 times in PBS.
2. Trypsinise 2-3 minutes.
3. Transfer colonies to 50 μl PBS and triturate until cells disaggregate.
4. Transfer PBS with cells to an electroporation cuvette containing 650 μl PBS and 100 μg DNA (in 100 μl PBS).
5. Leave cuvette on ice for 10 minutes.
6. Electroporate at 0.8 kV, 3 μF, time constant = 0.1.
7. Leave cuvette on ice for 10 minutes.
8. Divide contents of cuvette into 4 wells (200 μl each) and add 300 μl medium 2 to each well.
9. Leave cells for 3 hours and change medium (using medium 2).
10. Leave overnight before handling further.

APPENDIX 8: Freezing and thawing of cells

1. Dislodge colonies of cells with a drawn pipette.
2. Transfer colonies quickly to a drop of PBS and then to a drop of 0.25 ml freezing mix.
3. Transfer the drop of freezing mix quickly to a cryotube containing 0.4 ml of freezing mix.
4. Transfer to -70° freezer for overnight freezing, then transfer to liquid N_2 freezer.

To thaw:

1. Put 4.5-9.5 ml of Medium into a 25 ml universal.
2. Thaw cryovial in a waterbath then pipette the contents of vial into the universal.
3. Centrifuge at < 1000 rpm (600-800 optimal) for 3-5 minutes.

4. Aspirate medium from DIA-M feeders.

5. Aspirate media from universal, leaving cell clumps behind.

6. Add 1 ml of Medium 2 to universal and gently resuspend.

7. Pipette cells onto feeders (at 0.5 ml per well) and return to culture.

Freezing mixture:

42% FCS

48% Medium 3

10% DMSO

APPENDIX 9: ESRF purification and assay

9.1 Preparation of ESRF-containing ammonium sulphate cut of conditioned medium

1. ESRF-producing adherent cell line (D7A3-PE) is grown to near-confluency in GMEM/10% FCS. Currently, approximately 30-40 large (175 sq.cm.) flasks are used.

2. Medium is removed, cells rinsed twice with PBS then returned to serum-free GMEM/Ham's F12 (1:1) supplemented with 10mM HEPES, 3 μM sodium selenite, 1 μg/ml transferrin and 1 μg/ml insulin. Cells are gassed and returned to incubator in this medium for 3-4h minimum, overnight if more convenient. This is an adaptation and washing step.

3. Medium is removed and replaced with fresh serum-free medium for conditioning over 3-5 days. 35 ml medium per flask.

4. Conditioned medium is harvested, centrifuged to remove detached cells, and filter-sterilised. Approximately 1000 ml of medium is collected before proceeding to step 5.

5. Conditioned medium is brought to 35% saturation with ammonium sulphate. Saturated ammonium sulphate is added at 5 ml/min with stirring, in the cold room and left for a further 60 min. The precipitated protein is pelleted by centrifugation and constitutes approximately 20% of the total protein in the conditioned medium (Bradford assay).

6. The precipitate is solubilised in 2M urea. The centrifugation (step 5) is done in polypropylene bottles and the precipitate ends up as a viscous smear down the length of the bottle. In view of this, the solubilisation is done on a rocking roller apparatus for 1h at room temperature. 15-20 ml in 6 bottles.

7. The solubilised material is finally dialysed overnight against tissue culture-grade PBS (in cold room) then frozen in aliquots.

9.2 Assay

A number of embryonic stem-cell lines have been used for assaying ESRF activity, most commonly:-

CP1	for general morphology and alkaline phosphatase histochemistry

COKO18 for general morphology and oct4 expression (detected by lacZ reporter activity).

The basic protocol is the same in each case.

1. ES cells are seeded on to gelatinised 24-well plates at a density of 5,000 cells per well in 0.5 ml GMEM/10% FCS. Antibiotic is used at an appropriate concentration (unless the ESRF sample is filter sterilised).

2. After 2-3h test samples are added to the wells and the plates returned to the incubator for 4 days.

3. After 4 days, the cells are stained for alkaline phosphatase activity (Sigma Alkaline Phosphatase Leukocyte kit, cat. no. 86-R) or ß-galactosidase activity (X-gal stain).

4. Cells and colonies are inspected for typical ES-cell phenotype:

   - small, round cells
   - compact, rounded colonies
   - high expression of alkaline phosphatase or expression of oct4 as reflected by ß-galactosidase activity.

   1 unit of ES cell activity is defined as the minimal amount which produces typical ES-cell phenotype in 0.5 ml assay at the 4d point as described above (n.b. at this concentration the majority of cell will not have ES-cell phenotype).

APPENDIX 10: Ultracentrifugation of PE-conditioned medium

1. PE-CM prepared as described (Appendix 9).

2. PE-CM centrifuged at 100,000 x g for 75 min at 4°C; (S = 106).

3. Supernatant (S1) removed and pellet (P1) solubilised in 0.5 ml 2M urea.

4. Supernatant (S1) re-centrifuged at 265,000 x g for 18 h; (S = 3.3).

5. sUPERNATANT (S2) removed and pellet solubilised in 0.5 ml 2M urea (P2).

6. S1, S2, P1 and P2 were desalted/exchanged into PBS on Pharmacia PD10 (S) or BioGel P6-DG (P) columns.

7. Samples were assayed for ESRF activity.

Results

S1 activity similar to PE-CM

S2 no activity detected

P1 a very small amount of activity (less than PE-CM)

P2 very strong activity

APPENDIX 11: Lectin-affinity chromatography of ESRF

1. 25-35% saturated ammonium sulphate cut of PE-conditioned medium was subjected to fluid-phase isoelectric focussing (Rotofor).

2. precipitated material (pI = 4.25-4.5) was solubilised in 2M urea.

3. solubilised material was exchanged into lectin buffer* on a Pharmacia PD-10 column.

4. 200 µl exchanged material was loaded on to columns containing soyabean lectin, lentil lectin or wheat germ agglutinin (equilibrated with lectin buffer), left for 30 min, then another 200 µl added and the column left in the cold overnight.

5. columns were washed through with 5 x column volumes of lectin buffer; washes were collected.

6. columns were eluted with 5 x column volumes competing sugar** in lectin buffer and the eluted material collected.

7. wash-through and sugar-eluted material was exchanged into 20mM sodium phosphate pH 7.3 and assayed for ESRF activity.

***Lectin buffer**	20mM sodium phosphate pH 7.3 1M NaCl 0.1mM CaCl2 0.1mM MnCl2	
****Sugars**	soyabean -	galactose
	lentil -	α-methyl mannoside
	wheat germ -	N-acetylglucosamine

Results

Activity retained and specifically eluted on soyabean and lentil lectin columns but not on wheat germ agglutinin.

CLAIMS

1. A cytokine designated ESRF and characterised by the capacity to inhibit differentiation of ES cells (i) in the absence of DIA/LIF and (ii) in the absence of cytokines which act through gp130 and (iii) in the absence of interaction with gp130.

2. A cytokine as claimed in Claim 1 which is capable of inhibiting differentiation of ES cells in the absence of interaction with LIF-receptor.

3. A cytokine as claimed in Claim 1 or Claim 2 wherein said inhibition of differentiation is effected by a mechanism which is distinct from gp130.

4. A cytokine as claimed in any preceding claim which is obtainable by culturing cell line D7A3-PE and recovering conditioned medium therefrom.

5. A cytokine designated ESRF which is obtainable by culturing cell line D7A3-PE and is characterised by

- the capacity to inhibit differentiation of ES cells
- being distinguishable from DIA/LIF
- being distinguishable from IL-6/sIL-6R
- being distinguishable from CNTF
- being distinguishable from oncostatin M
- being distinguishable from cardiotrophin-1

6. A cytokine as claimed in Claim 5 wherein said inhibition of differentiation is effected by a mechanism which is distinct from gp130.

7. A cytokine according to Claim 5 or Claim 6 characterised in that its capacity to inhibit differentiation of ES cells cannot be eliminated by neutralising anti-DIA/LIF antiserum.

8. A cytokine according to any of Claims 5 to 7 characterised in that its capacity to inhibit differentiation of ES cells cannot be eliminated by neutralising anti-IL-6 soluble receptor antiserum.

9. A cytokine according to any preceding claim characterised in that its capacity to inhibit differentiation of ES cells cannot be eliminated by neutralising anti-CNTF antiserum.

10. A cytokine according to any preceding claim characterised in that its capacity to inhibit differentiation of ES cells cannot be eliminated by neutralising anti-gp130 antiserum.

11. A partially purified composition or a component thereof obtainable from the supernatant of DIA/LIF deficient cells and comprising at least one polypeptide having the capacity to inhibit differentiation of ES cells (i) in the absence of DIA/LIF and (ii) in the absence of cytokines which act through gp130 and (iii) in the absence of interaction with gp130.

12. A partially purified composition as claimed Claim 12 which capable of inhibiting differentiation of ES cells in the absence of interaction with LIF-receptor.

13. A partially purified composition or a component thereof according to Claim 11 or Claim 12 and additionally having at least one of the identifying characteristics specified in any one of Claims 2 to 10.

14. A purified polypeptide component of a composition according to any of Claims 11 to 13.

15. A method of producing a polypeptide having the capacity to inhibit differentiation of ES cells (i) in the absence of DIA/LIF and (ii) in the absence of cytokines which act through gp130 and (iii) in the absence of interaction with gp130, or a partially purified composition containing such a polypeptide, which comprises subjecting the supernatant of DIA/LIF deficient cells to a purification procedure whereby to isolate said polypeptide.

16. A method of propagating ES cells which comprises propagating the cells in the presence of the cytokine designated ESRF.

17. A method according to Claim 16 wherein the ES cells are propagated in the presence of ESRF in combination with DIA or other cytokines which act through gp130.

18. A method of establishing ES cells which comprises culturing cells derived from embryos in the presence of ESRF.

19. A method according to Claim 18 wherein the cells are cultured in the presence of ESRF in combination with DIA or other cytokines which act through gp130.

20. A method of establishing ES cells which comprises culturing primordial germ cells in the presence of ESRF.

21. A method according to Claim 18 wherein the cells are cultured in the presence of ESRF in combination with DIA or other cytokines which act through gp130.

22. A method according to any of Claims 16 to 21 wherein the cells are rodent cells.

23. A method according to any of Claims 16 to 21 wherein the cells are derived from a livestock species.

24. A method according to any of Claims 16 to 21 wherein the cells are derived from a primate species.

25. A method according to any of Claims 14 to 22 wherein the propagated cells bear a stem cell selectable marker such as Oct-4*neo* or Oct-4ßgeo.

26. A method of propagating somatic stem cells, such as haemopoietic stem cells, which comprises culturing said cells in the presence of ESRF.

27. The cell line designated D7A3-PE (ECCAC 94111845).

28. An established line of embryonic stem cells characterised by possessing at least five and preferably at least seven of the following features:

(i) the characteristic morphology of stem cells, including growth in clumps as small tightly packed cells with a high nuclear to cytoplasmic ratio,

(ii) at least one expression of specific markers selected from (a) alkaline phosphatase, (b) stage-specific embryonic antigen-1, (c) Oct-3/4,

(iii) non-expression of differentiation markers, for example H19 RNA,

(iv) substantial or unlimited propagation potential,

(v) stability to freezing and thawing,

(vi) stable euploid karyotype,

(vii) propagation dependent on cytokines,

(viii) in vitro differentiation induceable by withdrawal of cytokine(s), aggregation or chemical differentiation inducers,

(ix) ability to form teratocarcinomas comprising derivatives of endoderm, mesoderm and ectoderm,

(x) ability to colonise and/or reconstitute host tissues through the production of somatic stem cells and functionally differentiated progeny,

(xi) ability to colonise host embryos with contribution of functional differentiated progeny to chimeras,

(xii) ability to produce functional gametes in chimeras and generation of viable offspring,

(xiii) ability to integrate exogenous DNA,

and further characterised in that said cell line has a karyotype other than mouse.

29. An established line of embryonic stem cells according to Claim 28 characterised by possessing at least five and preferably at least seven of the specified features (i) to (viii).

30. An established line of embryonic stem cells according to Claim 29 further characterised by possessing at least one of the specified features (ix) to (xiii).

31. An established line of embryonic stem cells according to Claim 29 further characterised by possessing at least two of the specified features (ix) to (xiii).

32. An established line of embryonic stem cells according to Claim 28 or Claim 31 having a demonstrated rat karyotype.

33. A method for assaying and/or detecting growth factors that affect differentiation by a mechanism that is distinct from mechanisms involving DIA/LIF and/or gp130 interactions, which comprises culturing ES cells in the presence of a sample to be assayed, and detecting variation in growth or differentiation compared to cells cultured in the absence of the sample, characterised in that the ES cells have an LIF-negative and/or rLIF-negative phenotype.

Figure 1

Response of *lif-r* -/- ES cells to cytokines

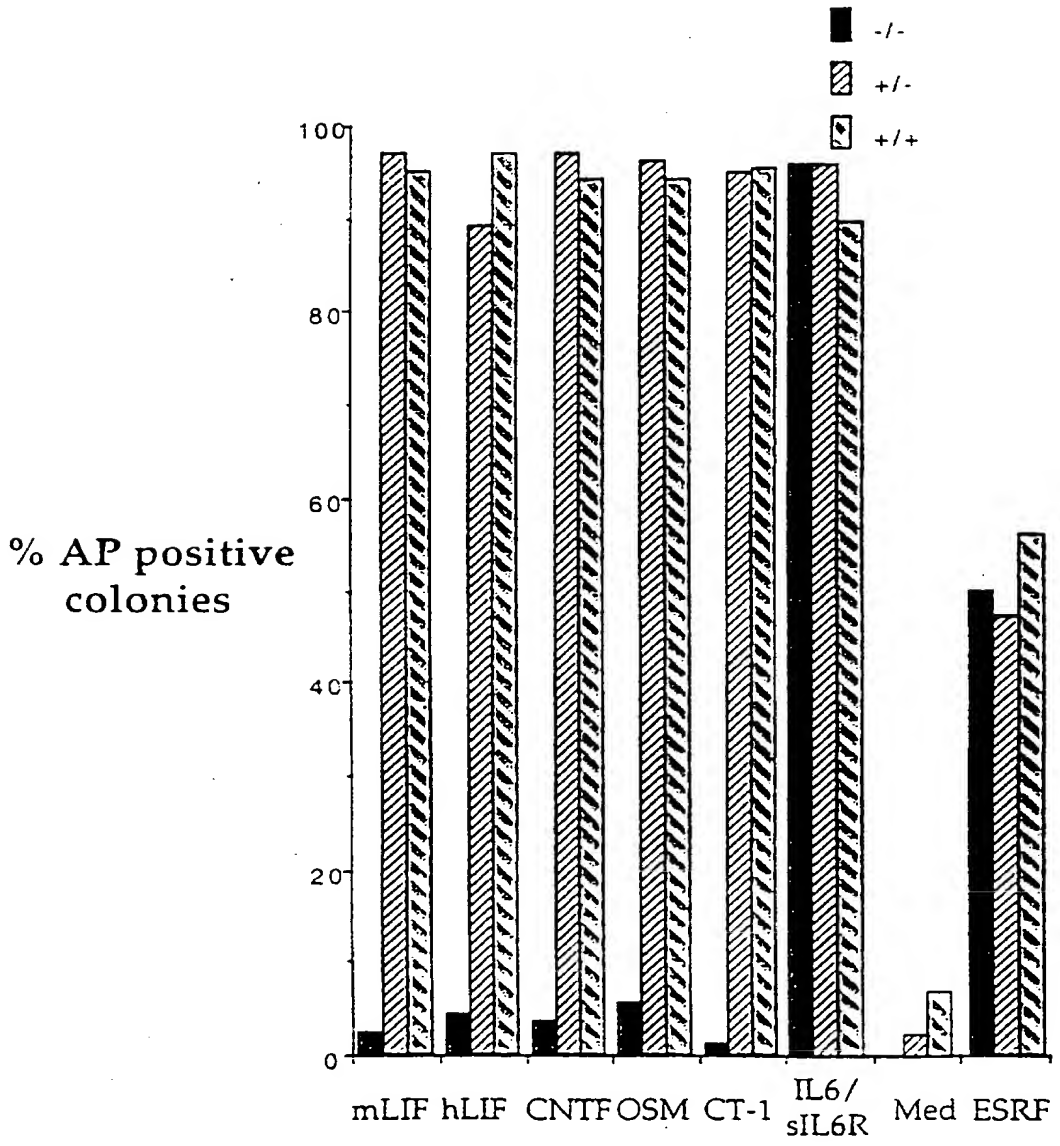


Figure 2

Phase contrast photomicrograph of rat ES cells

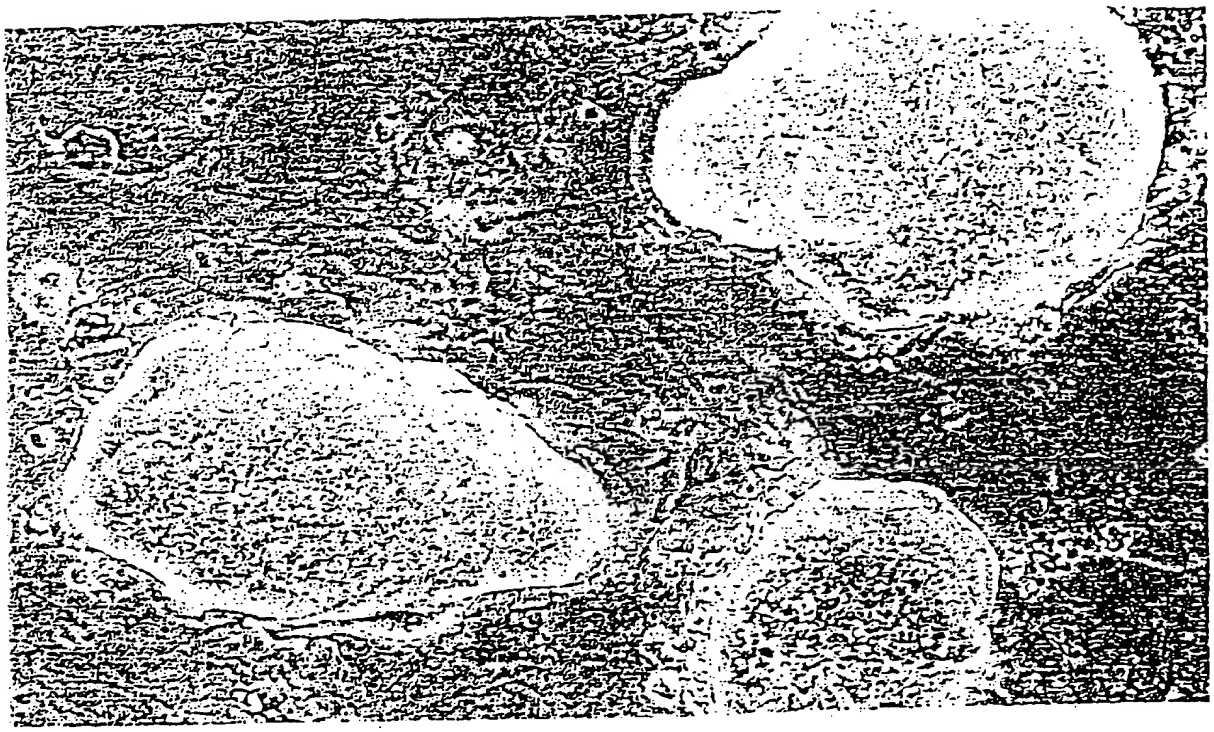


Figure 3

Metaphase chromosome spread from rat ES cells

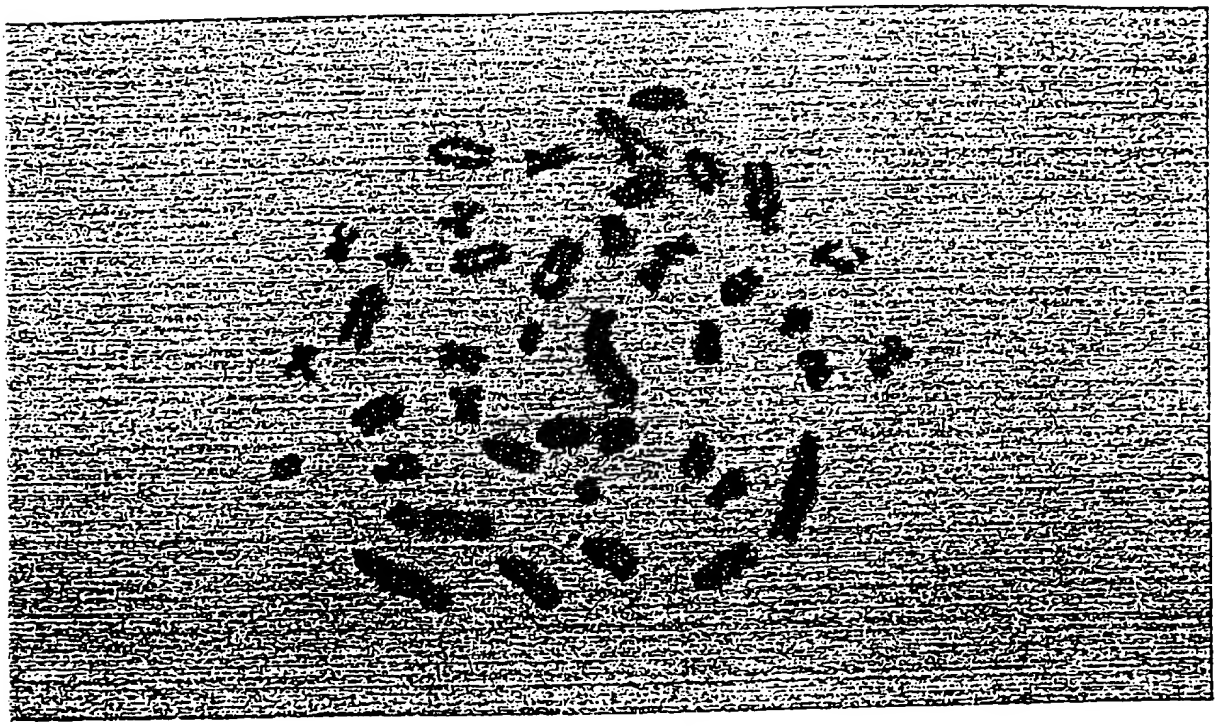
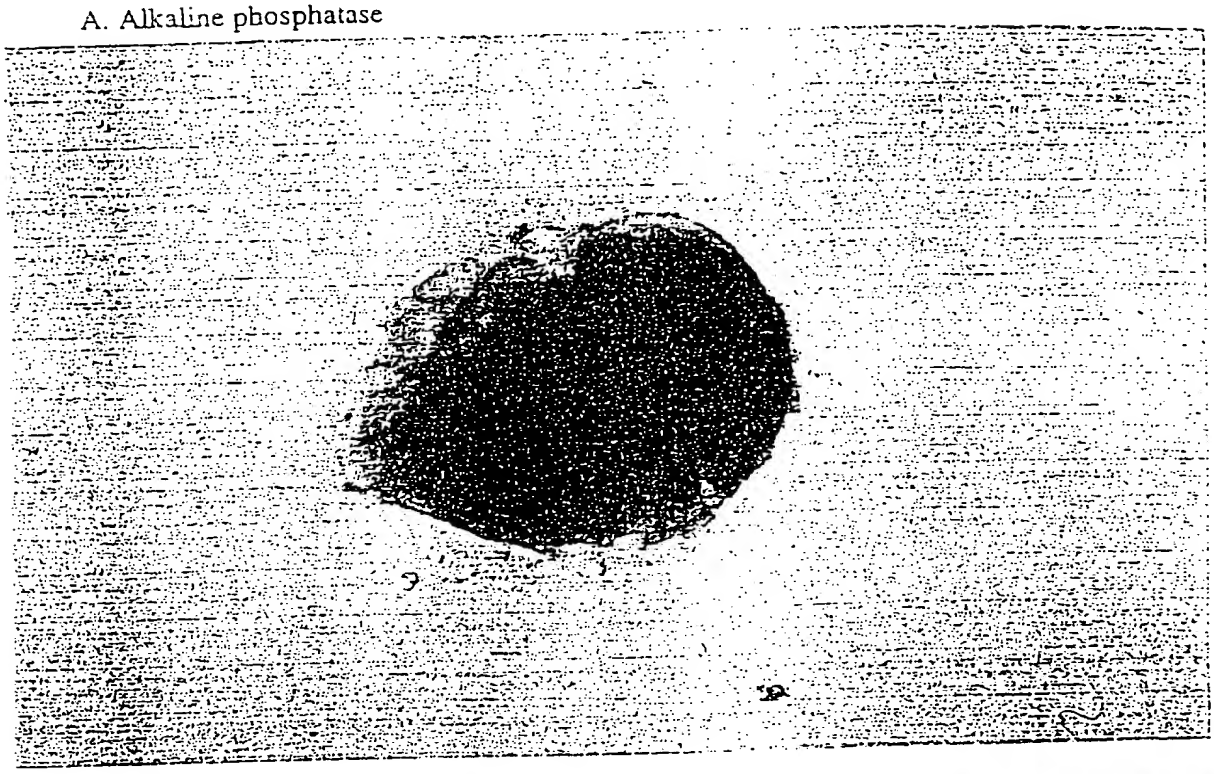


Figure 4

Marker expression in rat ES cells

A. Alkaline phosphatase



B. Oct-4 immunostaining

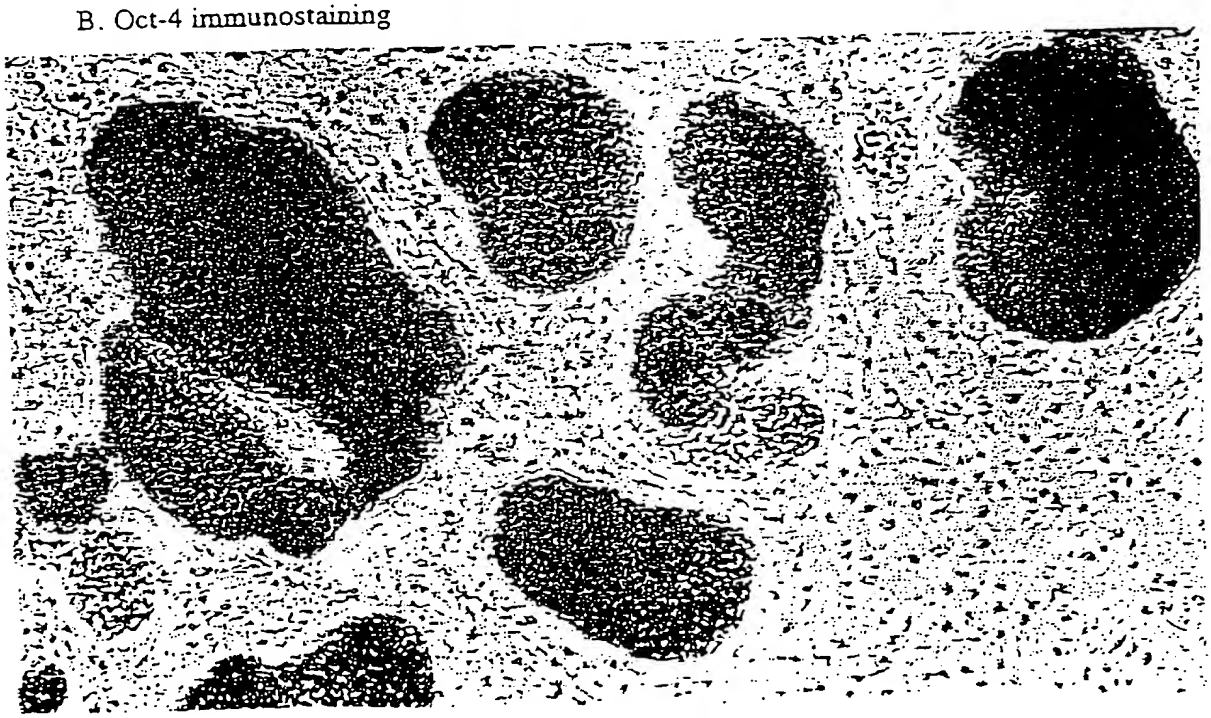
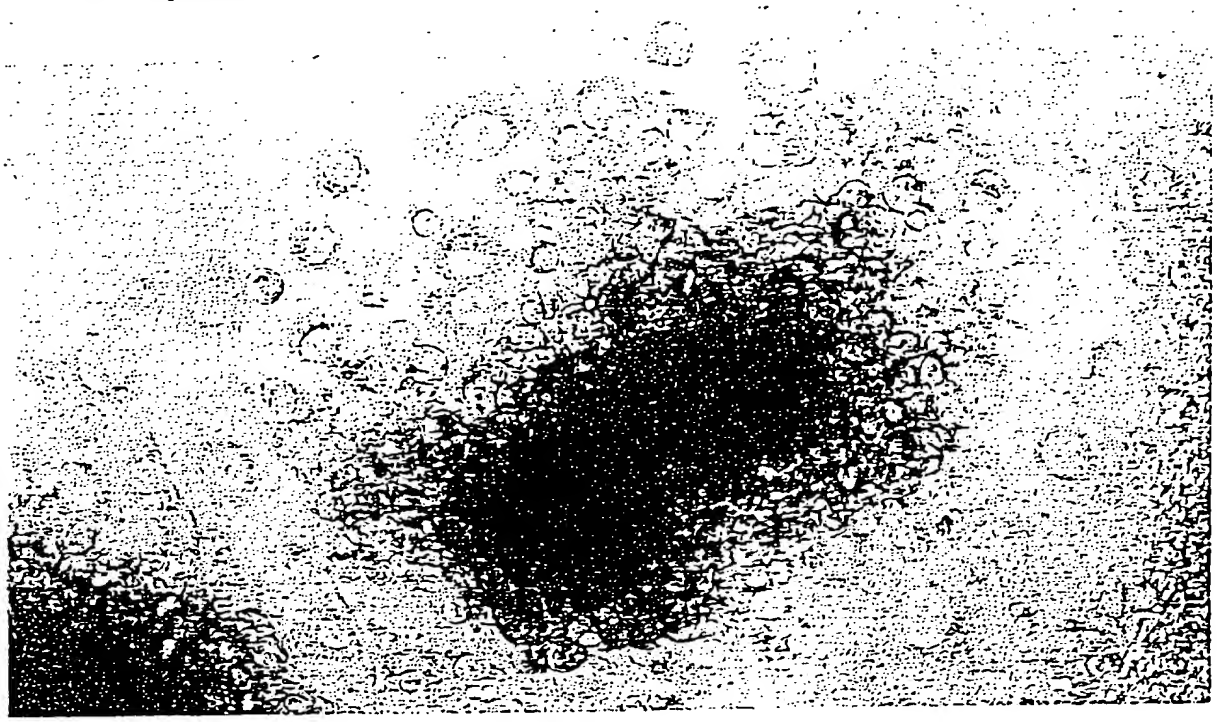


Figure 5

Differentiation of rat ES cells

A. Trophoblast



B. Parietal endoderm

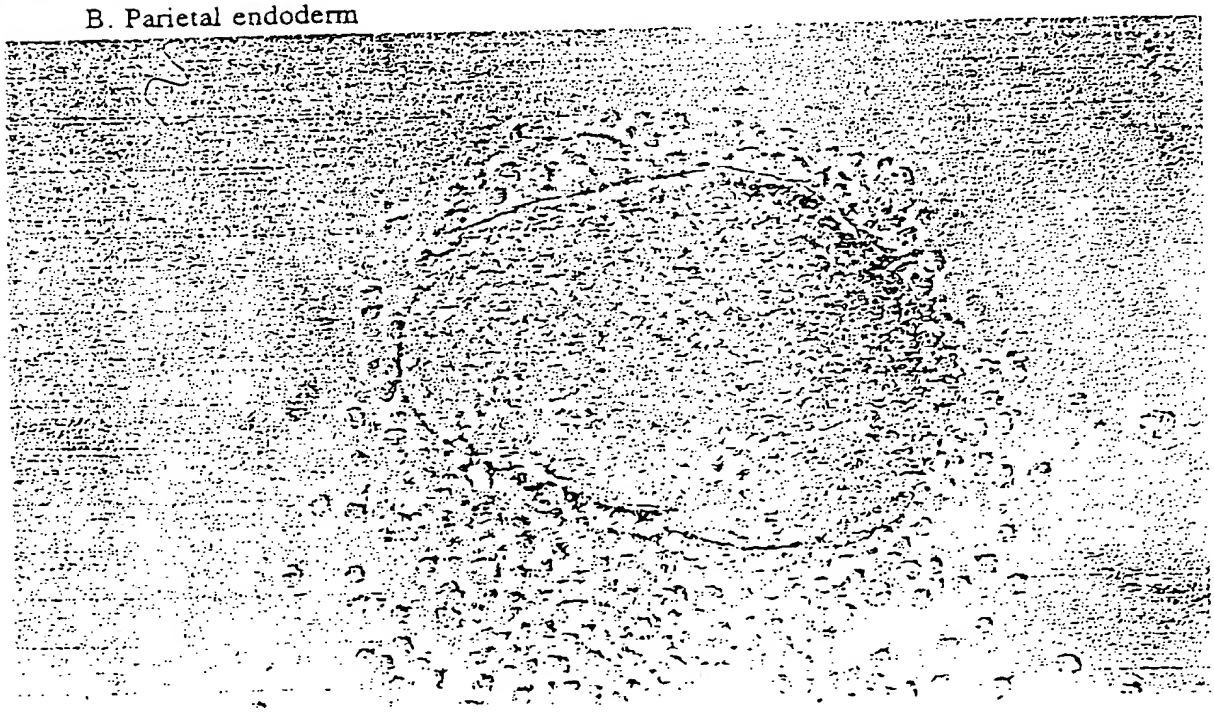
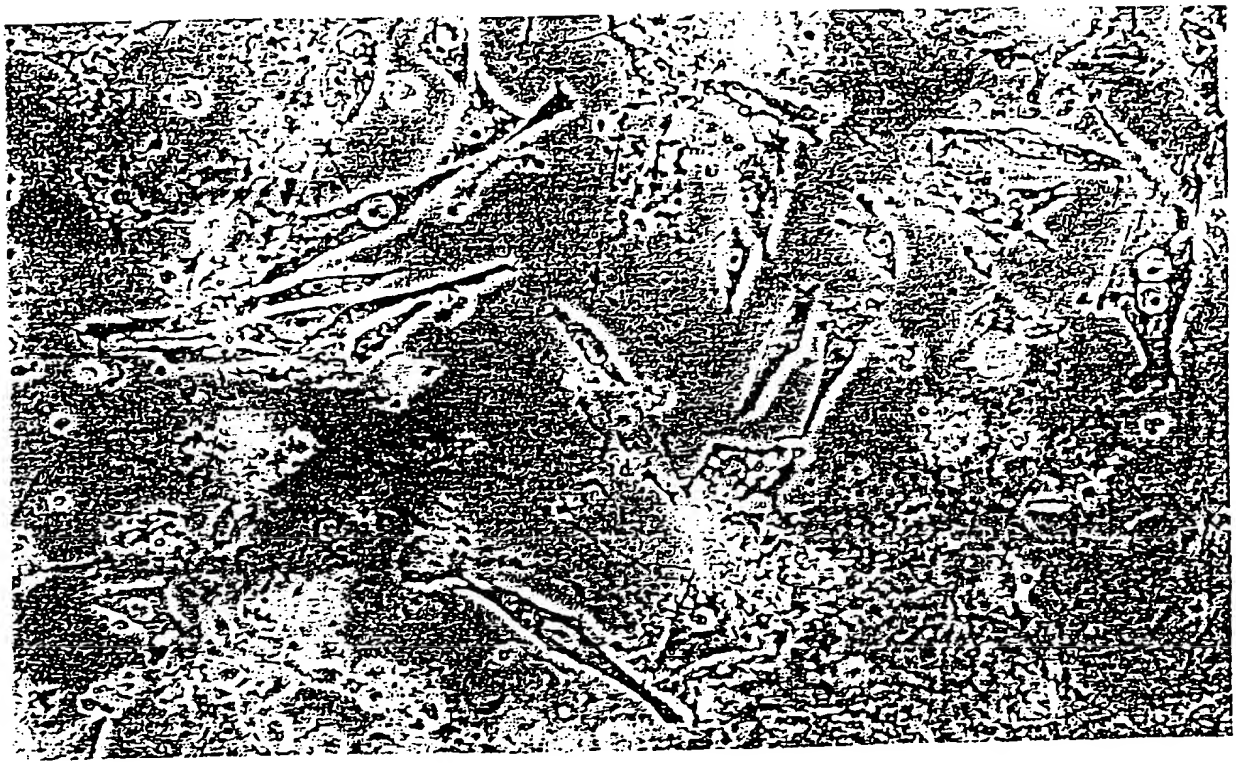


Figure 5 contd

Differentiation of rat ES cells

C. Bipolar cells



INTERNATIONAL SEARCH REPORT

In. ational Application No
T/GB 97/00426

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/01 C07K14/52

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12N C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA, vol. 92, no. 17, August 1995, pages 7844-7848, XP000604992 THOMSON J A ET AL: "ISOLATION OF A PRIMATE EMBRYONIC STEM CELL LINE" cited in the application see page 7846, right-hand column, paragraph 2 - page 7848, left-hand column, paragraph 2 --- -/--	1-14,24

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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1

Date of the actual completion of the international search	Date of mailing of the international search report
28 May 1997	05.06.97

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+ 31-70) 340-2040, Tx. 31 651 epo nl, Fax (+ 31-70) 340-3016	Fuhr, C

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INTERNATIONAL SEARCH REPORT

International Application No
/GB 97/00426

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	ONCOGENE, vol. 9, no. 12, December 1994, pages 3509-3517, XP000673923 S. MACLEAN-HUNTER ET AL.: "Expression of a RLF/L-MYC minigene inhibits differentiation of embryonic stem cells and embroid body formation" see page 3511, left-hand column, last paragraph - page 3516, left-hand column, paragraph 3	1-14,22
A	DATABASE WPI Section Ch, Week 9517 Derwent Publications Ltd., London, GB; Class B04, AN 95-127351 XP002031782 & JP 07 051 060 A (KISHIMOTO C) , 28 February 1995 see abstract	1-14
A	DATABASE WPI Section Ch, Week 9351 Derwent Publications Ltd., London, GB; Class B04, AN 93-408317 XP002031783 & JP 05 304 951 A (NT SCI KK) , 19 November 1993 see abstract	1-14
A	WO 90 02183 A (GENETICS INST ;INST NAT SANTE RECH MED (FR)) 8 March 1990 see page 6, line 5 - line 24; claims; examples	1-14
P,A	DATABASE WPI Section Ch, Week 9634 Derwent Publications Ltd., London, GB; Class B04, AN 96-336571 XP002031784 & JP 08 154 681 A (ITO HAM KK) , 18 June 1996 see abstract	1-14

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INTERNATIONAL SEARCH REPORT

Information on patent family members

Int. .onal Application No
PCT/GB 97/00426

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9002183 A	08-03-90	NONE	